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Perspective

TGF-β: the missing link in $CD4^+CD25^+$ regulatory T cell-mediated immunosuppression

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Abstract

A unique population of $CD4^+$ T lymphocytes that constitutively express CD25 has been recognized as anergic/suppressor cells. While the immunosuppressive activity of these $CD4^+CD25^+$ cells has been validated and implicated in tolerance, autoimmunity, transplantation, cancer and infectious diseases, the mechanism(s) by which they function still remains controversial. Although the involvement of TGF-β was initially discounted, emerging evidence now links this cytokine with $CD4^+CD25^+$ T cell-mediated suppression of antigen-activated T cells. In this perspective, we summarize recently published studies, as well as our own data, which shed light on how cell membrane-bound TGF-β can deliver a regulatory signal to target cells via a contact-dependent process. Moreover, suppressor T cell function is a complex process, tightly regulated by multiple factors, including IL-2, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and glucocorticoid induced TNF receptor (GITR). Collectively, multiple previously unconnected puzzle pieces are beginning to be linked into a more coherent, albeit incomplete picture of $CD4^+CD25^+$ T cell-mediated suppression.
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Following the original identification of a small, but powerful, population of $CD4^+CD25^+$ T cells which can dramatically suppress immune responsiveness, there has been an enormous effort to dissect the mechanisms by which these cells exert their suppressive prowess. In a short period of time, $CD4^+CD25^+$ regulatory T cells have generated so much interest in immunology because of their recognized importance in tolerance, autoimmune diseases, transplantation, cancer and infectious diseases. Nonetheless, the mechanisms underlying their ability to suppress immunity remain ill defined and hotly contested. New studies are providing pieces to this perplexing puzzle, including a role for TGF-β, and are linking several controversial and previously unconnected results into an emerging coherent picture.

First described by Sakaguchi et al. [1], these regulatory T cells, with their potent suppressive activity on normal responder T cell function in vivo and in vitro, are CD4 positive and constitutively express CD25 (IL-2 receptor alpha chain) on their cell surface. Produced in the thymus, approximately 5–10% of the $CD4^+$ T cells exiting the thymus belong to this phenotype (Fig. 1). $CD4^+CD25^+$ T cells circulate in watchful anticipation of an inflammatory/immune response where they will become activated to exhibit their suppressive potential. Functionally, these suppressor T cells are anergic and require T cell receptor (TCR) activation and cell contact for optimal inhibition of responder ($CD4^+CD25^-$) and self-reactive T cells, as they orchestrate peripheral tolerance.

Although most investigators agree that cell contact between suppressor and responder T cells is central to suppressor activity, the underlying molecular events remain a point of contention. Several studies have demonstrated that $CD4^+CD25^+$ T cells produce elevated levels of TGF-β in both mice and humans. Furthermore, independent studies [2–4] have demonstrated that anti-TGF-β1 antibody can reverse the suppression by $CD4^+CD25^+$ T cells in a dose dependent manner. A key to understanding the cell contact-dependent immunosuppression by the suppressor T cells was the recognition that the $CD4^+CD25^+$ T cells express surface membrane-bound TGF-β. Not only do $CD4^+CD25^+$ regulatory T cells express latent TGF-β [3], but these cells also bear TGF-β in its active configuration on the cell surface [4]. A defining piece of the puzzle was inserted when it was shown that enhanced TGF-β signaling receptors reside on the membrane of these cells. Thus, this population of cells constitutively expresses the signaling TGF-β receptor type II (TβRII) (Chen et al., submitted for publication), in addition to increased TGF-β, which underscores the potential for autocrine and/or paracrine receptor–ligand interactions (Fig. 2). Since the primary effect of TGF-β-TβRII signaling in T cells is to block IL-2

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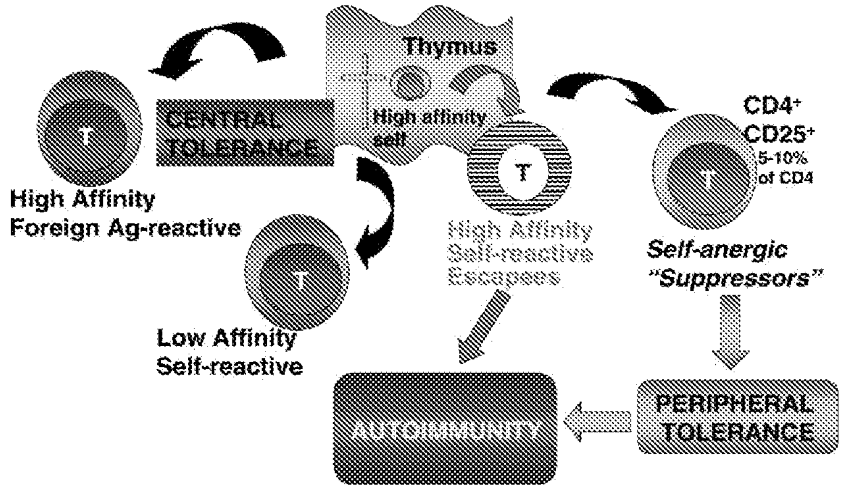

Fig. 1. Peripheral immunologic self-tolerance maintained by $CD4^+CD25^+$ "anergic/suppressor" T cells. A small number of $CD4^+$ T cells constitutively expressing CD25 (5–10% of total $CD4^+$ T cells) are generated in the thymus, and migrate into the periphery to regulate normal immune responses and maintain self-tolerance. Although high affinity self-reactive T cells are typically deleted in the thymus, some may escape to the periphery. In cooperation with activation induced T cell apoptosis, anergy and suppressive cytokines, $CD4^+CD25^+$ suppressor T cells regulate the balance between immunity and tolerance to safeguard the host against autoimmunity and immunopathology.

production [5–8], the coordinate expression of both cell surface-associated active TGF-β and TβRII on the suppressor cells may explain why these cells are functionally anergic and lack IL-2 expression upon TCR stimulation. Moreover, fulfilling another tenet of suppressor T cell function, TCR engagement augments the expression of both TGF-β and TβRII, enhancing this self-inflicted anergy.

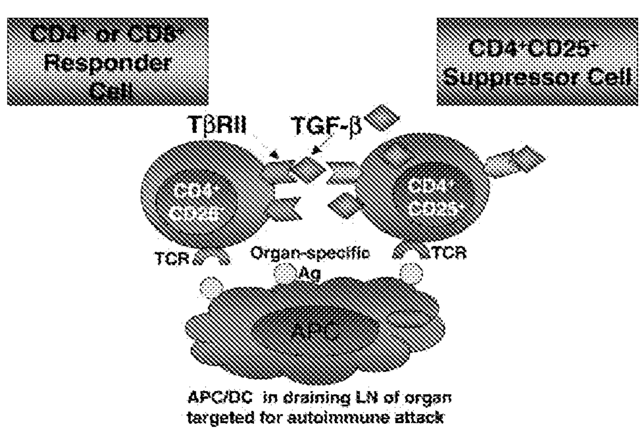

Fig. 2. Mechanism of $CD4^+CD25^+$-mediated immunosuppression. $CD4^+CD25^+$ T cell suppression requires T cell receptor (TCR) engagement and cell–cell contact with the target cells. Once activated, $CD4^+CD25^+$ cells inhibit $CD4^+CD25^-$ responder T cells and/or other cells (e.g. B cells) in a non-specific manner, which can be reversed by high dose IL-2 or anti-CD28 stimulation. Freshly isolated $CD4^+CD25^+$ T cells express cell surface TGF-β and TGF-β receptor II (TβRII), which are upregulated by TCR stimulation in the presence of antigen presenting cells (APCs). Naïve $CD4^+CD25^-$ responder T cells express neither TGF-β nor TβRII, but become positive for TβRII after TCR stimulation. The co-expression of TGF-β and TβRII on $CD4^+CD25^+$ cells may be responsible for their anergic state. The interaction between surface TGF-β on $CD4^+CD25^+$ suppressor and TβRII on $CD4^+CD25^+$ responder may provide the molecular mechanism by which the "suppressors" function.

While the existence of TGF-β on the surface of $CD4^+CD25^+$ T cells offers a clue for the generally recognized cell-contact dependence and antigen non-specificity features associated with suppressor T cell function [9,10], it does not address the mechanism whereby TGF-β drives the suppression of TβRII-negative responder T cells. TGF-β is a cytokine with known involvement in the regulation of growth inhibition, apoptosis, differentiation and/or suppression of multiple cell types [11–13], yet the documented requirement for cell contact in T cell suppressor activity initially appeared incompatible with this soluble cytokine. Very compelling then was the unanticipated demonstration of upregulation of TβRII on the responder cells following triggering of their TCR, thereby establishing a connection through which suppressor cell derived TGF-β can bind to activated responder cells and transduce contact-dependent suppressive signals [4].

TGF-β mediates its effects on cells through a heteromeric complex consisting of type I (RI) and type II (RII) receptor components. TβRI and TβRII are serine–threonine kinases that phosphorylate downstream signaling proteins once TGF-β binds to TβRII, which in turn recruits and phosphorylates TβRI. The receptor complex then propagates the signal through the phosphorylation of cytosolic proteins, including the Smad proteins, Smad2 and Smad3 [14] (Fig. 3). Once activated, Smad2 and Smad3 form a hetero-oligomeric complex, typically including the co-Smad4, and translocate to the nucleus where they modulate transcription of specific genes through *cis*-regulatory Smad-binding sequences and through binding with other transcription factors [15,16]. Of significance was the evidence that $CD4^+CD25^+$suppressor T cells induce increased phosphorylation of Smad2/3 in $CD4^+CD25^-$ responder T cells upon cell contact (Chen et al., submitted for publication), confirming engagement

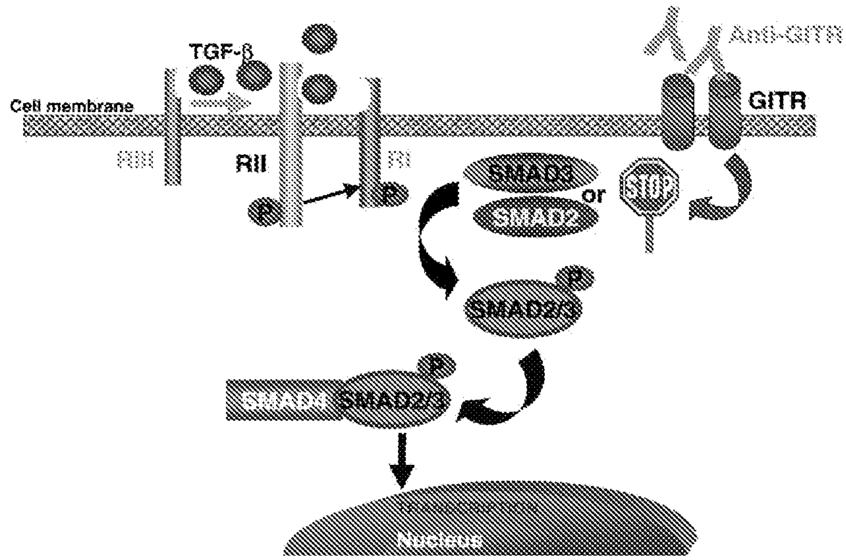

Fig. 3. TGF-β signaling pathway and role of GITR to reverse suppression. TGF-β binds its receptor II (RII), potentially in the context of receptor III (RIII), to phosphorylate TGF-β receptor I (RI). This ligand–receptor complex initiates a signaling cascade involving phosphorylation of Smad2 and/or 3(P-Smad2/3). In complex with the common Smad4, P-Smad2/3 translocates to the nucleus to regulate gene transcription. $CD4^+CD25^+$ suppressors also express GITR on their membranes and engagement of this receptor with anti-GITR antibody antagonizes TGF-β signal transduction by inhibiting Smad2/3 phosphorylation to block suppression.

of the TGF-β-TβRII signaling pathway. Moreover, recent studies have revealed that multiple intersecting pathways may contribute to the TGF-β signaling cascade [15]. Consistent with involvement of multiple pathways, mice lacking only Smad3 survive much longer than mice deficient in the ligand, TGF-β1 (TGF-β1−/−), which develop rapid and massive T cell activation and inflammation culminating in their early demise. Since Smad3 null $CD4^+CD25^-$ responder T cells are inhibited directly by exogenous TGF-β (Chen et al., unpublished data), to the same extent as wildtype $CD4^+CD25^+$ suppressor T cells [17], it is likely that, at a minimum, Smad2, in addition to Smad3 is engaged in the process of suppression. Nonetheless, the finding that responder T cells become TβRII positive upon activation provides the missing link by which suppressor T cell-associated TGF-β interacts with and engages its cognate receptor on the responder T cells to efficiently block IL-2 production and proliferation. Moreover, the upregulation of membrane active TGF-β and TβRII on the suppressor cells after TCR and antigen presenting cell (APC) stimulation [4] provides insight into the necessity of activation for the suppressor cells to function optimally [9,10]: a minimal binding threshold between TGF-β on the suppressor and TβRII on the responder may be required to deliver a sufficient negative signal to thwart IL-2 production and the proliferative response.

Since $CD4^+CD25^+$ T cells express surface TGF-β, implicated in the suppression mechanism, one might expect to see that freshly isolated $CD4^+CD25^+$ T cells from TGF-β1−/− mice have no suppressive effect. Indeed, TGF-β1−/− $CD4^+CD25^+$ T cells in vitro do not inhibit responder cells at normal suppressor/responder ratios (Chen et al., unpublished data). However, with increasing numbers of TGF-β1 null $CD4^+CD25^+$ T cells (5–10-fold), some inhibitory activity can be detected (unpublished), as recently reported [17], implicating potential alternative pathways of suppression. In this regard, the phenotype of freshly isolated $CD4^+CD25^+$ TGF-β1−/− T cells is distinct from their wildtype counterparts. The majority of TGF-β1−/− $CD4^+CD25^+$ T cells are $CD62L^{low}$ (a phenotypic marker of activated/memory cells) compared to only a small percentage of wildtype cells, reflecting their constitutive activation [18]. This phenotype of $CD4^+CD25^+$ T cells is seen in 6–7-day-old TGF-β1−/− mice prior to overt evidence of their autoimmune-like inflammation [19]. Clearly, the TGF-β null $CD4^+CD25^+$ T cell populations are unique and/or may express alternative regulatory molecules. Although there are temporal, spatial and functional differences between TGF-β1 and its homologs, TGF-β2 and TGF-β3, in vivo, both TGF-β2 and TGF-β3 exhibit suppression in vitro and may play a role in the absence of TGF-β1. A compensatory increase in other immunoregulatory factors, such as IL-10, must also be considered. In the co-culture system, the hypersensitivity of TGF-β1−/− T cells to apoptosis [19] may trigger wildtype APCs to release TGF-β [20], which may contribute to suppression. Collectively, the jury is still out on how TGF-β null T cells promote suppression. Additionally, the ability of high numbers of $CD4^+CD25^+$ TGF-β1−/− T cells to effect suppression must be viewed with caution since maternal transfer of TGF-β [21] and its passive binding to these cells cannot be excluded. Nonetheless, as clearly observed in vivo, in the absence of TGF-β,

there is insufficient suppression, and the mice succumb to fatal autoimmune-like disease.

Multiple factors may impact on the ability of $CD4^+CD25^+$ suppressor/anergic T cells to orchestrate suppression of responder and/or autoreactive T cells. Among these is IL-2, since culture IL-2 levels determine how or if TGF-β affects T cell proliferation [5,22], and high doses of IL-2 and/or anti-CD28 stimulation reverse $CD4^+CD25^+$ T cell anergy and suppressive activity [23]. In this same vein, our findings that exogenous IL-2 does not affect cell surface-associated TGF-β on suppressor $CD4^+CD25^+$ T cells clarifies the paradox that high dose IL-2 can reverse their anergic state, but preserve their suppressive ability [9,10].

Another molecule which appears to impact on TGF-β-mediated suppression is cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), a negative co-stimulatory molecule for T cell activation, constitutively expressed in $CD4^+CD25^+$ T cells [24]. Although two initial reports provided evidence that CTLA-4 engagement is responsible for $CD4^+CD25^+$ T cell function [2,25], the molecular mechanisms by which CTLA-4 signaling contributes to the suppression remain unknown. The functional link between CTLA-4 signaling and TGF-β production in $CD4^+$ T cells was first documented in 1998 [26], has been confirmed in murine and human $CD4^+$ T cells [27–29], and was recently associated with $CD4^+CD25^+$ T cells [3]. Linking these observations, we demonstrated that CTLA-4 engagement upregulates cell surface active TGF-β and TβRII expression on $CD4^+CD25^+$ suppressor T cells [4]. In a surprising observation, we also found that CTLA-4 on the responder T cells contributes to the suppressive sequelae, since blocking CTLA-4 also downregulates TβRII on activated $CD4^+CD25^-$ responder T cells (Chen et al., submitted for publication). This scenario may mitigate responder cell vulnerability to suppression. Although the mechanism by which CTLA-4 regulates TGF-β and TβRII expression remains an unresolved, but captivating question, a functional linkage between CTLA-4 and TGF-β signaling is consistent with the known negative CTLA-4 signal in the regulation of $CD4^+$ T cells.

The powerful activity of these $CD4^+CD25^+$ regulatory cells necessitates a counterbalance, and recent studies [30,31] have identified a surface molecule, the glucocorticoid induced TNF receptor (GITR), which may serve this purpose. Reportedly, engagement of the GITR on $CD4^+CD25^+$ T cells abrogates their suppressive ability. Signaling rather than blocking of GITR on $CD4^+CD25^+$ suppressor T cells appears responsible for disengaging suppression, since an anti-GITR antibody, which engages the receptor, enhances IL-2 induced-proliferation of $CD4^+CD25^+$ T cells [31], without affecting $CD4^+CD25^-$ T cells. Not surprisingly, in light of the dominant role for TGF-β in suppression, reversal of $CD4^+CD25^+$ T cell anergy and suppression invoked by engagement of GITR involves an intermediate step of inhibition/prevention of TGF-β signaling as manifested by blunted Smad2/3 phosphorylation (Fig. 3) (Chen et al., submitted for publication). While still mechanistically enigmatic, the inhibition of $CD4^+CD25^-$ responder T cell IL-2 production by suppressor cell membrane-bound TGF-β and/or other factors such as TNF-related activation induced cytokine and receptor activator of NFκB (TRANCE-RANK) [32], T cell negative regulator Tob [33], Th1 transcription factor T-bet [34] and IL-10 [35] may impact on or modify the outcome of GITR signaling of $CD4^+CD25^+$ T cells. A likely cross-talk between GITR and Smad2/3 signaling offers provocative insight into the balance between T cell activation and suppression.

In short, the emerging data link multiple unconnected puzzle pieces into a more coherent, albeit yet incomplete picture of $CD4^+CD25^+$ T cell-mediated suppression. $CD4^+CD25^+$ suppressor, but not $CD4^+CD25^-$ responder T cells, not only express cell surface TGF-β, but also TβRII, a key receptor for TGF-β signaling. On the other hand, the responder T cells become TβRII-positive after TCR stimulation, thereby becoming susceptible to TGF-β upon cell contact as manifested by enhanced phosphorylation of Smad2/3. Collectively, these findings provide molecular evidence in support of the accepted unique features of $CD4^+CD25^+$ suppressor T cells, including anergic to TCR stimulation, cell contact dependency, antigen non-specific suppression and a requirement for TCR engagement. In addition, exogenous IL-2 reverses anergy without altering surface TGF-β to preserve $CD4^+CD25^+$ suppressive ability. While CTLA-4 engagement promotes suppression by enhancing cell surface-associated TGF-β and TβRII, GITR stimulation serves as a counterbalance, inhibiting TGF-β-mediated signaling to blunt immunosuppression. As these pieces are fit into the puzzle, our understanding of the fundamental mechanisms by which $CD4^+CD25^+$ T cells orchestrate suppression and mediate immune tolerance evolves. Since characterization of the $CD4^+CD25^+$ suppressor T cells appears central to defining aberrancies in autoimmunity, transplantation, cancer and HIV/AIDS, continued exploration of this population will uncover potential regulatory pathways in the treatment of multiple immunopathological conditions.

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